Short communication

Lectin coated MnO2 nanoparticle: its antileishmanial activity and toxicity

López-Abente and Nuriaiop Aragonés

Hematology Department,Hospital Clinic Barcelona, IDIBAPS., Barcelona, Belgium

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* Corresponding Author's E-mail: LópezassAbente@yahoo.com

Materials and methods

All materials which used in this research was analytical grade, and were used without any purification. MnO2 nanoparticles were sourced from Lolitech Co., Germany and L.major promastigotes (MRHO/IR/75/ER) were obtained from Center for Research and Training in Skin Disease and Leprosy, Tehran University of Medical Sciences, Tehran, Iran. Also, Balb/c mice were provided from Pasteur Institute of Iran. Giemsa, methanol, trypsin enzyme, RPMI 1649, FCS, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide or MTT, N-1- naphtyl- ethylene diamine, acidic sulfanilamide, nitrite sodium, H2O2, TMB, HRP enzyme, and HBSS were purchased from Sigma-Aldrich Chemical Co, (St Louis, MO). In this study, LDH activity was quantified by LDH kit, which was from Novin Gostar Co.

Preparation and characterization of lectin coated MnO2 nanoparticles

In the first step, MnO2 nanoparticles were ball milled 10 minutes and sterilized at 121 ºC for 20 minute. Then, MnO2 nanoparticles at concentration of 1000 µg/ml were prepared in distilled water, and incubated with PNA lection solution at concentration 2 mg/ml for 2 hours at 37 ºC. Then, the suspension was centrifuged at 10000 rpm and the supernatant was resuspended in PBS. To confirm attachment of lectin to MnO2 nanoparticles, fourier transform infrared spectroscopy (FTIR)(ELICO, India) was used. The wide range concentration of coated nanoparticles including 1000, 500, 250, 125, and 62 µg/ml was prepared and used in this research. Also, the same range of concentration was prepared for naked MnO2 nanoparticles and PNA lectin. The structure and size of coated and naked MnO2 nanoparticle was investigated by dynamic light scattering (DLS)(Malvern Instruments, Italy) and scanning electron microscopy (SEM)(Hitachi S-2400). The

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average hydrodynamic diameter of coated and naked MnO2 nanoparticle was read after triplicate tests by DLS analysis at 37 ºC. Also, SEM analysis was carried out after coating with a thin layer of gold by sputtering, and with 15 KV as accelerating voltage.

mouse peritoneum

Preparation L.major promastigotes, peritoneal macrophage cells, and skin cells

Firstly, L.major promastigotes (MRHO/IR/75/ER) were incubated in RPMI-1649 medium with L-glutamine enriched with 10% fetal calf serum (FCS) for 72 hours at 37 ºC and sub cultured weekly. The quantity of promastigotes was 3×10^5 per one ml of medium. On other hand, 10 Balb/c mice (18-20 g) were selected, and 5 ml of RPMI medium were injected to mice peritoneum. Then, peritoneal macrophage cells were aspirated and washed with hanks' balanced salt solution (HBSS). The washed macrophage cells were added to RPMI1649 medium supplemented with 10% FCS until reached to 5×10^5/ml. To investigate infectivity of promastigotes, macrophages adhered to the solid support. So, the macrophage cell suspension was added to 96-well plates, incubated at 37 ºC for 24 hours, and rinsed with RPMI-1640 medium for removing non-adherent cells. For preparation of mouse skin cells, one peace of dorsal skin at size of 2×2 cm was cut, and rinsed in phosphate-buffered saline (PBS). Then, 5 ml of trypsin enzyme at concentration of 1 mg/ml were added to skin peaces, and incubated at 37°C for 45 min. In the next step, skin pieces which treated with enzyme were crushed mechanically, and centrifuged at 3000 rpm for 20 minutes. Finally, isolated skin cells were washed and added to RPMI1649 medium, which the final cell concentration was reached to 10^6cells/ml.

The evaluation of cytotoxicity

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MTT assay was used for cytotoxicity evaluation of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and lectin PNA. As mentioned prior, different concentrations of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin including 1000, 500, 250, 125, and 62 µg/ml were prepared, and then separately incubated with L.major promastigotes, peritoneal macrophage, and skin cells for 24 h at 37°C. All cells were washed with PBS after 24 h incubation. Then, 100 µL of RPMI1649 medium and 25 µL of MTT solution (5 mg/ml) were added, and incubated 3 h at 37°C. The quantity of formazan formation was read by microplate reader (Novin Gostar, Iran) at 490 nm, and cell viability was measured according to optical densities (OD) of each well and its control.

The study of LDH quantification

To investigate the cell membrane injury after exposure of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin, the quantity of LDH enzyme in promastigote medium was measured using LDH kit. After 24 h exposure, cell suspension was centrifuged and 10 µL of supernatant were added to 1 ml of reagent 1 and 2, which contained lactate and NAD, respectively. Then, mean OD of each well were read at 340 nm by microplate reader (Novin Gostar, Iran), and LDH quantities were calculated according to standard curve.

The evaluation of parasite proliferation

All concentrations of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin were exposed to L. major promastigote suspension for 24, 48, and 72 h at 27°C, and the quantity of promastigote cells after these incubation times was counted by neubauer lam (Marienfeld, Germany). This test was carried out three times, the mean± SD was reported, and compared with control group.

The evaluation of promastigote infectivity

For this test, in the first step, L.major promastigotes were exposed to different concentrations of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin for 24 h at 27°C. Then, to remove free or non-bonded nanoparticles, all parasites washed with PBS. In the next step, parasites incubated with peritoneal macrophage cells at 37°C for 5 hours. To remove non-phagocytized promastigote, centrifugation at 1000 rpm was done, and finally infected macrophage cells incubated at 37°C for 72 hours. After incubation, macrophage cells were stained with Giemsa, and by an optical microscopy (magnification 400X), infected macrophage percent was calculated and compared with control. On the other hand, the average number of parasites per macrophage was counted, and infection index was calculated according to formula 1.

Formula1.

Infection index= the average number of amastigotes per macrophage × the infected macrophage percentage

The evaluation of nitrite and H2O2 produced by infected macrophage

To evaluate the activity of macrophage, the quantity of nitrite and H2O2 in supernatant of macrophage medium was measured after exposure parasites, which treated with different concentrations of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin. According to griess test, quantification of nitrite ion was carried out. First, 50 µL of supernatant were added to 50 µL of acidic sulfanilamide, and incubated at 25°C for 5 min. Then, 100 µL N-1-naphtyl- ethylene diamine were added, and again incubated at 25°C but for 15 minutes. In final step, OD of each well was read at 550 nm and nitrite concentration was measured by standard

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curve. The quantification of H2O2 produced by macrophages was done. Briefly, 50 µL of cell supernatant were added to 100 µL of TMB, and incubated at 25°C for 5 minutes. Then, 50 µL of HRP enzyme were added, and again incubated at 25°C for 5 minutes. like griess test, after reading OD of each well at 630 nm, H2O2 concentration was calculated using standard curve.

Statistical analyzing

All tests were done in three separate times and results reported as mean± standard deviation (SD). To detect significant differences between tested groups and control, student's t-test was carried out in SPSS software (v 19.0 for Windows; SPSS Inc, Chicago, IL), and P- values less than 0.05 considered as statistical significance.

Characterization of coated and naked MnO2 nanoparticle

Characterization of both lectin coated and naked MnO2 nanoparticle were done by three methods including SEM, DLS, and FTIR. Figure1 shows the results of three characterization methods. SEM results demonstrated that size of naked MnO2 nanoparticle (about 20 nm) was smaller than lectin coated MnO2 nanoparticle (about 30 nm). Also, the average hydrodynamic diameter of naked nanoparticle and coated nanoparticle were 45 nm and 65 nm, respectively. FTIR results showed the presences of amide band I (1635 cm^{-1}), amide band II (1535 cm^{-1}), and amine group band (3300-3500 cm^{-1}) in lectin coated MnO2 nanoparticle, which indicated the lectin adsorption by MnO2 nanoparticle.

Cytotoxicity evaluation

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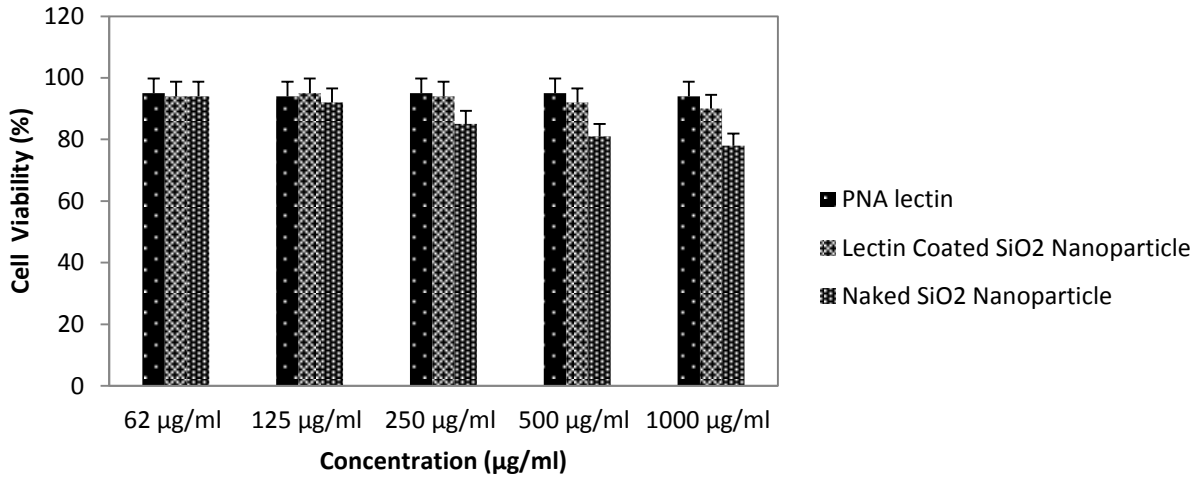
The cytotoxicity evaluation of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and lectin PNA were carried out by MTT assay after 24 h incubation at 37°C. According to figure 2, PNA lectin had no toxicity on L.major promastigote compared to control (P<0.05). Also, lectin coated MnO2 nanoparticle had a little toxic effect only at concentration of 500 and 1000 µg/ml, the related cell viability was 92 and 90, respectively. On the other hand, the most cytotoxicity, with 78% cell viability, was observed for naked MnO2 nanoparticle at concentration of 1000 µg/ml. There were significant differences between related cell viability of naked MnO2 nanoparticle and lectin coated or PNA lectin alone at concentration of 250, 500, and 1000 µg/ml (P<0.05).



LDH quantification

The cell membrane damage after exposure of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin was studied by quantification of LDH enzyme in parasite medium supernatant. As shown in figure 3, PNA lectin and lectin coated MnO2 nanoparticle had no effect on promastigote membrane with no significant difference compared to control (P>0.05), but naked MnO2 nanoparticle had some effect on membrane at two final concentrations (500 and 1000 µg/ml)

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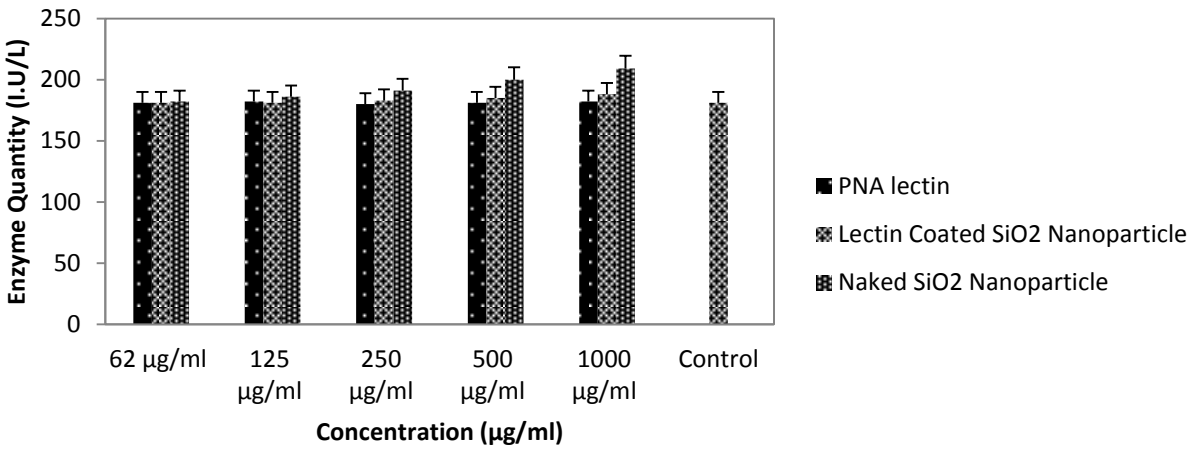
with significant difference (P<0.05). The maximum LDH was 209 I.U/L at concentration of 1000 µg/ml.



Promastigote proliferation

To study the effect of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin on cell cycle and proliferation of promastigote, the number of parasite was recorded after 72 h exposure. Figure 4 shows that like MTT and LDH test, PNA lectin are not effective on proliferation of promastigote. Also, both lectin coated MnO2 nanoparticle and naked MnO2 nanoparticle at concentration of 500 and 1000 µg/ml decrease proliferation of promastigotes gradually. This test showed that proliferation of promastigote was more decreased by naked nanoparticle than lectin coated nanoparticle. Furthermore, the minimum parasite number (7.2×10^6 /ml) was observed for naked nanoparticle at concentration of 1000 µg/ml. There was significant difference between quantities of parasite after exposure of lectin coated MnO2 nanoparticle and naked MnO2 nanoparticle at concentration of 500 and 1000 µg/ml (P<0.05).

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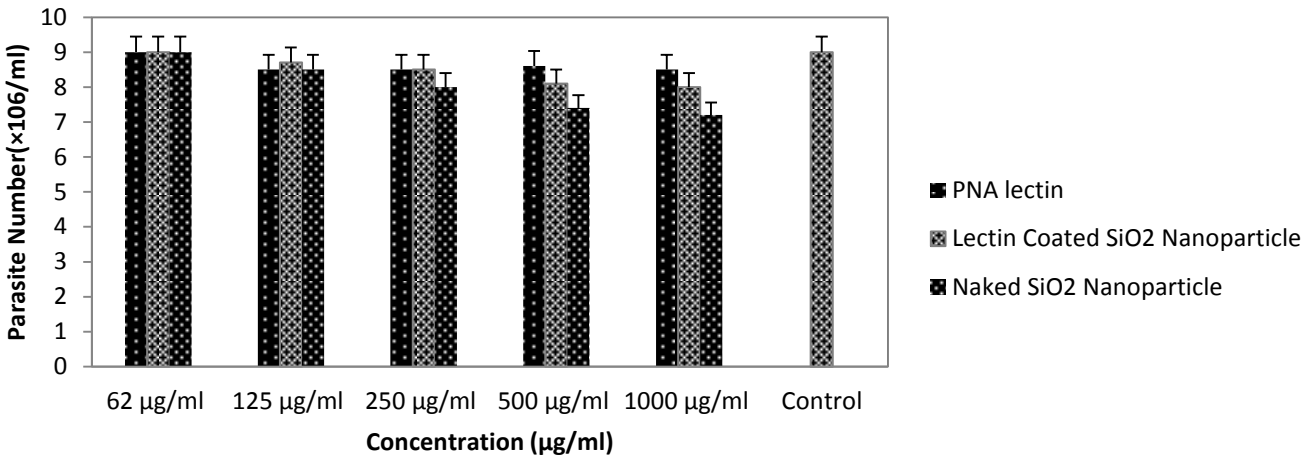

Promastigote infectivity and infection index

In this research, the ability of treated promastigotes for infecting of macrophage was measured by two parameters including the percentage of infected macrophage and infection index, which shown in figure 5 and 6, respectively. These tests showed that although lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin decreased both infectivity and infectivity index, but lectin coated MnO2 nanoparticle was more powerful, with the least infectivity (32%) and infectivity index (50%). The quantity of infectivity and infectivity index for control were 93% and 280%, respectively. There are significant differences between infectivity of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin at all concentration (except at concentration 62 µg/ml) and control group ($P<0.05$). Also, such significant differences were observed to infectivity index for all ($P<0.05$). The trend of infectivity and infectivity index were dose dependent for lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin.

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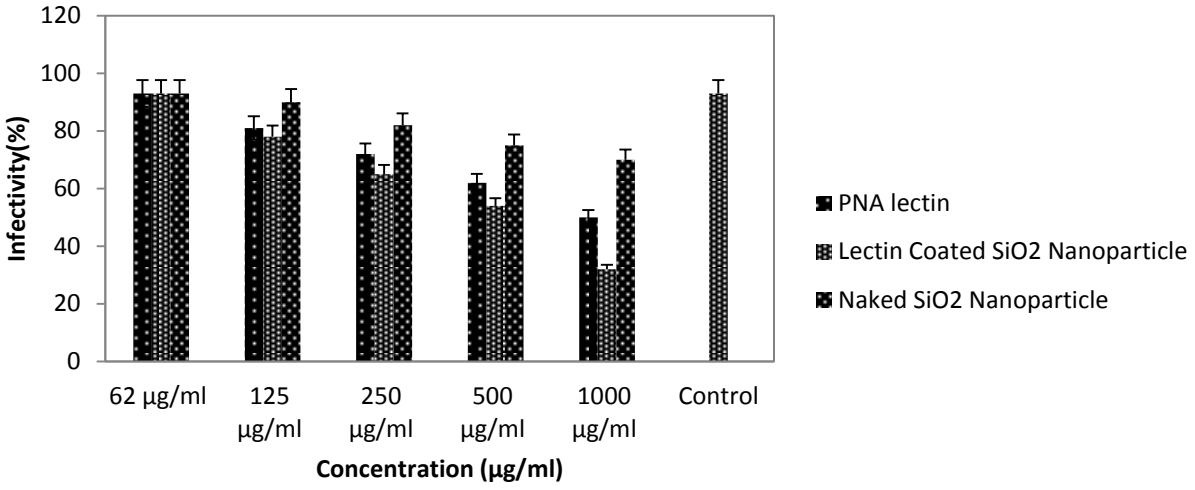

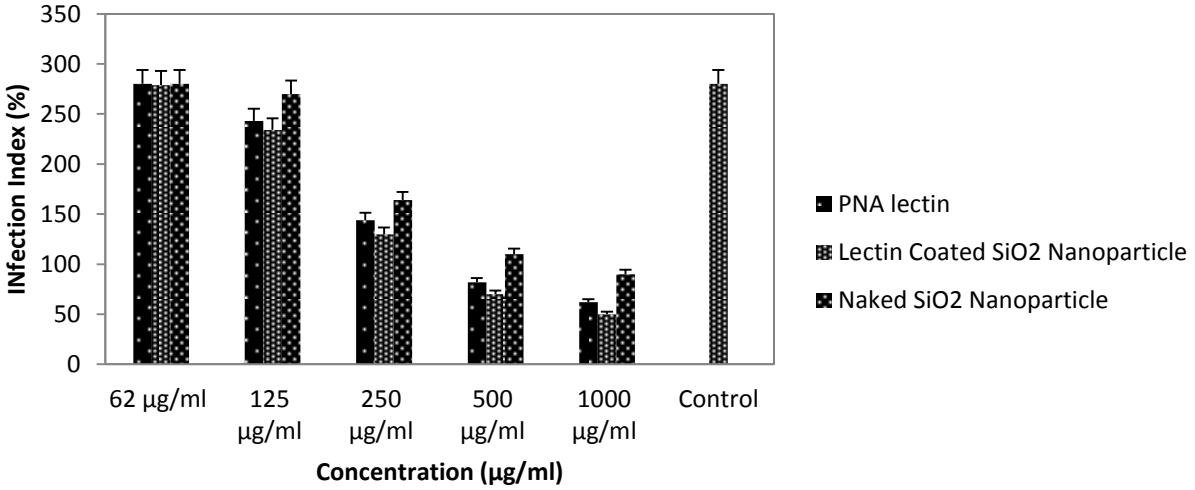

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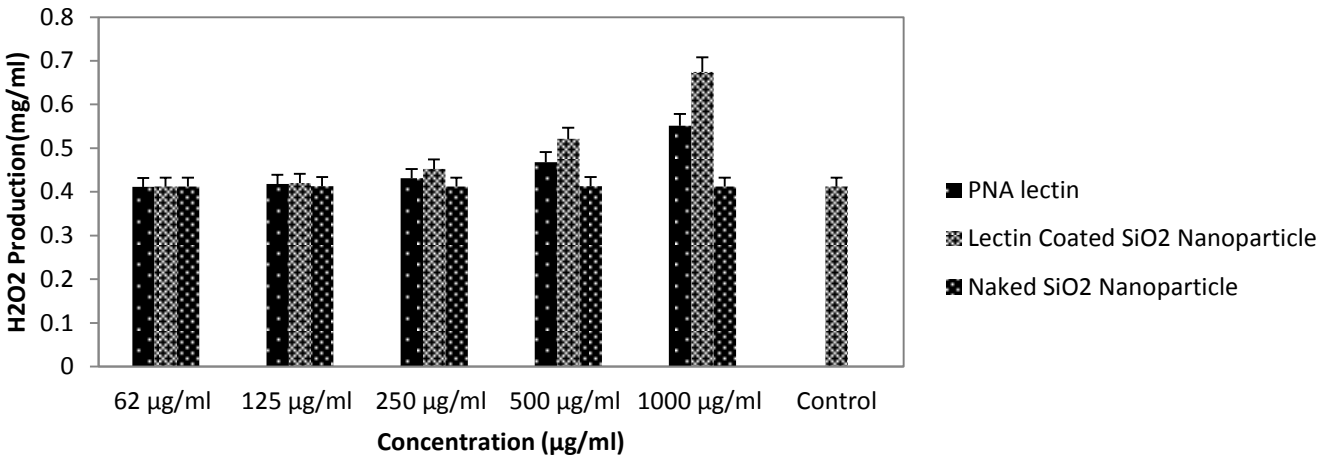
Nitrite and H2O2 production by infected macrophage

In this study, activity of macrophage was evaluated by measurement of H2O2 and nitrite concentration in macrophage medium. As mentioned prior, parasites firstly incubated with different concentrations of lectin coated MnO2 nanoparticle, naked MnO2 nanoparticle, and PNA lectin, then exposed to macrophages. After 72 h incubation, the quantity of nitrite and H2O2 were measured. Figure 7 and figure 8 show the concentration of H2O2 and nitrite after 72 h incubation with parasite. As shown, naked MnO2 nanoparticle did not affected production of H2O2 and nitrite compared to control (P>0.05), but lectin coated MnO2 nanoparticle and PNA lectin affected H2O2 and nitrite production at concentration of 250, 500, and 1000 µg/ml with significant differences compared to control (P<0.05). It is seen that lectin coated MnO2 nanoparticle is more powerful in macrophage activation than PNA lectin. The most concentration of H2O2 and nitrite were 0.674 mg/ml and 70 µM, respectively for lectin coated MnO2 nanoparticle.



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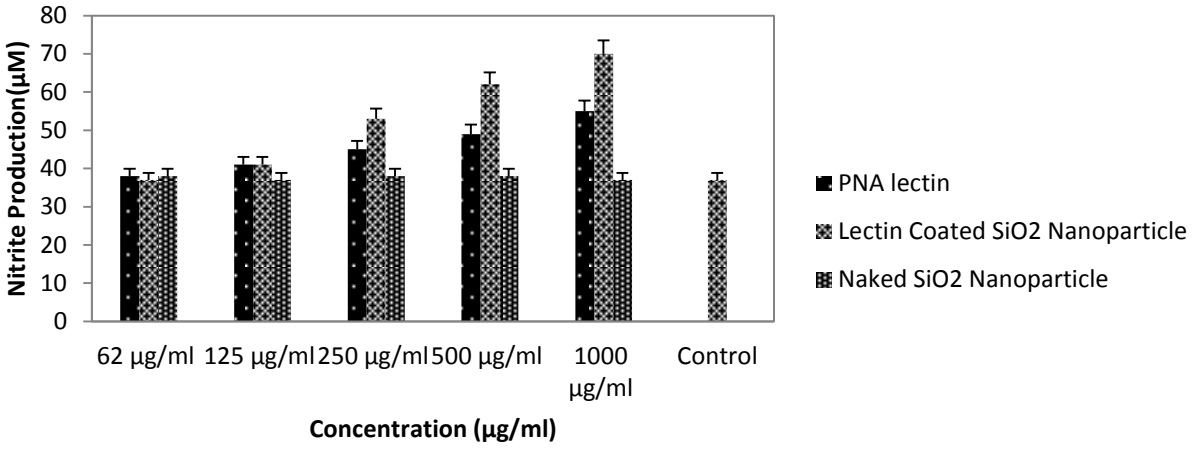

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